DIFFUSION PROFILES OF Na^+-FLUORESCEIN IN FROG VENTRICULAR MUSCLE

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ABSTRACT Frog ventricular muscle strips were placed in a single sucrose-gap chamber to measure the interdiffusion of solutes across the sucrose-Ringer's solution partition. Steady-state diffusion profiles of fluorescein sodium developed along the axis of the muscle in the physiological node by continuously perfusing the sucrose pool with 210-mM sucrose plus fluorescein (5–10 mM). Fluorescein was found to diffuse freely through the extracellular space of the ventricular muscle without binding to the tissue. The fluorescence of Na^+-fluorescein in the muscle (measured at 530 ± 30 nm) varied linearly with the dye concentration in the sucrose perfusate. The diffusion profiles of dye in the test node depended on the tightness or snaring of the muscle strip by the latex diaphragms, the diameter of the muscle strip, and changes in hydrostatic pressure between the sucrose and Ringer's solution pools. Fluorescein concentration in the cross section of test node closest to the latex partition (sucrose-Ringer's solution interface) ranged between 4–13% of the dye concentration in the sucrose pool. These values are more than five times smaller than those estimated theoretically, assuming free diffusion. The experimental findings indicate that the presence of a physical barrier, such as a rubber diaphragm, limits free interdiffusion of solutes across the sucrose gap. The presence of such a barrier thus prevents large concentration gradients from occurring in the extracellular spaces along the physiological node.

INTRODUCTION

The sucrose gap has been extensively used as a node to pass the intracellular current and to voltage-clamp myocardial preparations (Morad and Trautwein, 1968; Beeler and Reuter, 1970; Rougier et al., 1968, 1969; Tarr and Trank, 1971). Theoretical analyses of the single and double sucrose-gap techniques have raised serious questions regarding the interpretation and validity of voltage-clamp data (Johnson and Lieberman, 1971; Atwell and Cohen, 1977). Despite these difficulties, the technique has been used widely and effectively to obtain detailed information regarding the ionic currents and excitation-contraction coupling events in the myocardium (Morad and Goldman, 1973).

An often stated criticism of the single sucrose-gap method in multicellular preparations is the possible mixing of the solutions at the sucrose-Ringer's solution interface. In chambers where the separation of fluids is achieved by rubber diaphragms or vaseline seals, solutes are thought to interdiffuse through the extracellular space and establish steady-state concentration gradients. Such nonideal separation of fluids at the sucrose-Ringer's solution interface is thought to generate inhomogeneities of potential and current distributions along the preparations. Measurements (Kleber, 1973) and mathematical modeling (Atwell and Cohen, 1977) have suggested that significant concentrations of solutes interdiffuse through the muscle making the physiological node spatially ill-defined. Such studies predict, for instance, that at the partition, preparations contain a 50:50 mixture of sucrose and Ringer's solutions, and 0.5 mm away from the interface contain a 20:80 mixture of the solutes.

In this report optical methods were used to measure the steady-state diffusion profiles of fluorescein sodium in frog ventricular strips in a single sucrose-gap chamber. Fluorescein was found to diffuse passively through the extracellular spaces of the tissue in a manner similar to sucrose or Na^+. Our results show that in muscle strips (0.5 mm in diameter), partitioned by rubber diaphragms that fit firmly around the muscle, the intermixing of solutes diffusing through the extracellular space is <5% across the interface. The results are not consistent with previous experimental and theoretical analyses (Kleber, 1973; Atwell and Cohen, 1977), but support more recent theoretical work that takes into account the squeezing factor imposed by the latex membrane on the diffusion of solutes through the extracellular space of the muscle (Lammel, 1981; Cleemann, L., and M. Suenson, manuscript submitted for publication).

MATERIALS AND METHODS

Ventricular Strip Preparation

Frog hearts (*Rana pipiens*) were excised and bathed in Ringer's solution in a paraffin dish. Thin muscle strips, ranging from 0.4 to 1.5 mm in diameter, were dissected from the basal region of the frog ventricle and

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placed in a single sucrose-gap chamber adapted for optical measurements (Morad and Salama, 1979).

Solutions

The physiological pool was perfused with an air equilibrated Ringer's solution at room temperature containing: NaCl, 118; $NaHCO_3$, 2; KCl, 3; and $CaCl_2$ all in mmol/l; pH 7.6–7.8. The sucrose gap was perfused with 210-mM sucrose, 0.01-mM $MnSO_4$ plus 0, 5, or 10-mM fluorescein sodium, and the KCl pool with 120-mM KCl and 2-mM $NaHCO_3$. All solutions were prepared with deionized and distilled water. The three solutions were allowed to flow continuously into the chamber under constant hydrostatic pressure and the levels of the fluids in the three compartments were separately adjusted by vacuum suction. Fluorescein sodium was purchased from Fisher Scientific Co., Allied Corp., (Pittsburgh, PA).

Sucrose-Gap Chamber

The Chamber consisted of three compartments (*a*) KCl, (*b*) sucrose, and (*c*) Ringer's solution pool separated by two 50-μm thick latex diaphragms. With the gap set at 2.5 mm, one end of the muscle was pulled by a thread through the diaphragm assembly. The gap length was then reduced to slacken the segment of muscle in the sucrose gap. The free end of the muscle in the physiological pool was attached to a tension transducer. The arrangement for the electronic and optical instrumentation was essentially similar to those described earlier (Morad and Orkand, 1971; Morad and Salama, 1979).

Fluorescence Measurements from the Test Node

Two glass windows on opposite sides of the physiological compartment were constructed to permit uniform illumination of the test node (0.5 to 2 mm in length) by an incident light beam. A third window at the bottom of the Ringer's solution compartment was within 3 mm of the muscle to achieve efficient collection of the fluorescent light from the test nodes at 90° from the excitation beam. Light from a water-cooled tungsten halogen lamp (Q6.6A/T2 1/2/Cl; General Electric Co., Lamp Components Sales Operation, Cleveland, Ohio) was passed through an interference filter (Omega Optical, Inc., Brattleboro, VT) transmitting at 450 ± 30 nm and illuminated the test node from a side window of the physiological compartment. From the bottom window, an image of the test node was projected, magnified by a factor of 10 and focused on a black aluminum screen held in place with a micromanipulator. A 250-μm slit in the screen oriented perpendicularly to the axis of the muscle strip allowed light from a 25-μm segment of the muscle to pass through an interference filter (520 ± 30 nm) and to fall on the surface of a photomultiplier tube (9524B EMI, Plainview, NY). The slit was displaced parallel to the axis of the muscle, in 1-mm steps in the image plane (corresponding to 100-μm steps on the muscle), to measure the scattered or fluorescent light from different segments of the test node.

Experimental Procedure

Ventricular strips were placed in a sucrose-gap chamber and were equilibrated for at least 30 min. Trans-gap action potentials of 80 mV or better were recorded. The diameter and length of the test node were measured using a micrometer scale in the objective of a dissecting microscope (Bausch & Lomb Inc., Instruments & Systems Div., Rochester, NY) and was magnified by a factor of 10 in the image plane where the slit was positioned. In the absence of fluorescein in the gap, the scattered light from various segments of the muscle varied by <10%. Precautions were taken to verify that the diameter of the test node was uniform and that the optical set-up detected light from various segments of muscle with equal sensitivity. After perfusing the gap with sucrose solution containing fluorescein sodium, a cone of green fluorescent light appeared in the test node within 2 min and its intensity remained stable for several hours. The fluorescence levels from the same cross sections of muscle were measured to obtain steady-state diffusion profiles of fluorescein in the test node. The highest fluorescence intensity from the test node was measured from the 25-μm segment of muscle nearest the sucrose-Ringer's solution partition. The fluorescent light was generally 30–45 times greater than the background light levels.

To determine the concentration of dye in the extracellular space of the test node from its fluorescence intensity, the physiological pool was continuously perfused with Ringer's solution plus various concentrations of fluorescein sodium for 15 min. The dye solution was rapidly removed by suction. The muscle was then flushed with dye-free solution to wash excess dye from the surface of the muscle. The fluorescence intensity was then recorded and was found linear with dye concentrations ranging from 0 to 10 mM. In a typical experiment (using 5-mM fluorescein in the gap), a cone of fluorescence protruded from the sucrose-Ringer's solution interface into the test node within 2 min of perfusion. After 15 to 30 min of equilibration, fluorescence levels were measured from 25-μm segments of test node spaced 100-μm apart. The fluorescence from each segment was recorded as a percentage of the fluorescence signal measured when the muscle in the physiological compartment was equilibrated in 5-mM fluorescein. The steady-state diffusion profiles were then plotted as the percentage of dye concentration in the test node vs. distance from the sucrose-Ringer's solution interface. To determine the 100% fluorescence level, the physiological pool was perfused with Ringer's solution plus 5-mM fluorescein for 15–30 min, emptied of fluid, and flushed once with dye-free solution. The fluorescence signals measured immediately after this procedure were considered equivalent to 5-mM dye in the extracellular spaces of the muscle or as the 100% fluorescence level. To confirm the validity of this procedure, at the end of each experiment (with fluorescein only in the gap) the 100% fluorescence level of the muscle was remeasured by sliding the muscle out of the sucrose gap into the physiological pool. The percentage of the fluorescence levels measured from segments

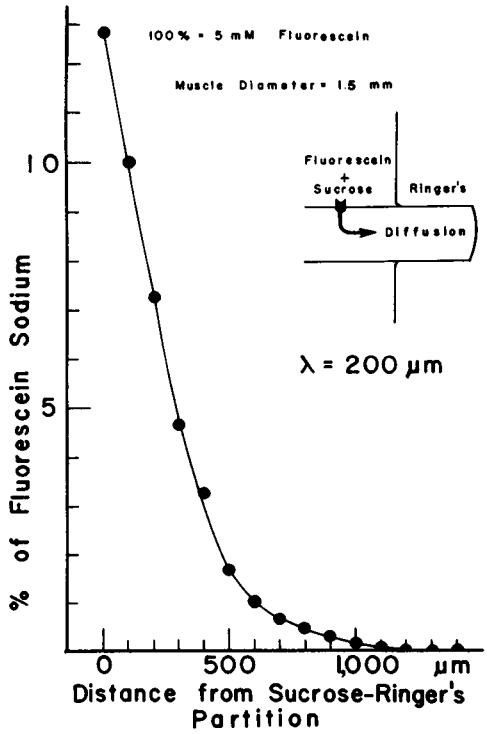

FIGURE 1 Diffusion profile of fluorescein in a frog ventricular muscle strip. The percent fluorescein concentrations (●) within 25-μm thick cross sections of the test node (1.5 mm in diameter) are plotted as a function of distance from the sucrose-Ringer's solution partition.

of muscle in the sucrose-gap were equal to measurements obtained by bathing the test node in 5-mM fluorescein.

RESULTS

In Fig. 1, the steady-state diffusion profile of fluorescein is shown for a 1.5-mm diameter muscle strip. Each data point represents the fluorescence intensity recorded from a 25-μm segment of muscle. The maximum fluorescence measured in the first 25-μm segment of the muscle in the test node (nearest to the gap) was 12.7% of the fluorescence level measured in the sucrose gap. In six muscle strips, 1.5-mm diameter, the maximum fluorescein concentrations near the interface ranged from 10 to 13%. Fluorescence levels decreased to half-maximal values 190 to 350 μm away from the interface.

In Fig. 2, the percentage of fluorescence concentration is plotted as a function of distance along the test node for 0.5 mm, 1.0 mm, and 1.5-mm diameter muscles. In 0.5-mm and 1-mm diameter strips, the maximum fluorescein concentration ranged from 4 to 6%, and 8 to 10%, and reached half-maximum values in 380–440 and 200–390 μm, respectively (see Table I).

The concentrations of fluorescein in the test node and

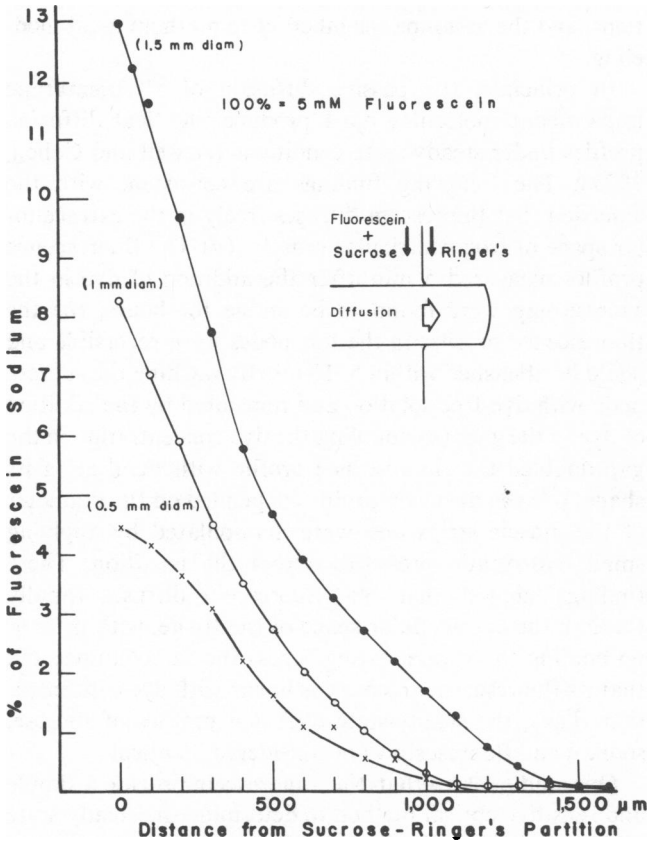

FIGURE 2 Diffusion profiles of fluorescein in frog ventricular muscle strips of varying diameter. The percent fluorescein concentrations within 25-μm thick cross sections of the test node are plotted as a function of distance from the sucrose-Ringer's solution partition for 1.5 mm (●), 1.0 mm (○), and 0.5 mm (×) diameter muscle strip.

TABLE I
STEADY-STATE DIFFUSION PROFILES MEASURED IN 22 VENTRICULAR STRIPS

Diameter of muscle strip	Maximum fluorescence (5-mM fluorescein in sucrose node)	Distance from the interface at half-maximum
mm	%	μm
0.5	4.5	420
	5.9	390
	4.8	380
	5.5	440
	4.4	450
	4.2	400
0.7	7.0	320
	8.2	260
1.0	8.1	230
	8.2	330
	8.3	340
	8.5	300
	8.7	390
	9.5	290
	9.8	200
	9.5	260
1.5	10.2	310
	10.7	205
	11.5	320
	11.9	290
	12.7	190
	12.9	350

the shape of the diffusion profile were also manipulated by applying a hydrostatic pressure across the sucrose-Ringer's solution partition. Raising (or lowering) the hydrostatic pressure by 3 mm of the fluorescein-labeled sucrose solution increased (or decreased) the maximal fluorescein concentration in the muscle bundle in the physiological solutions at the interface by 2–3% (Fig. 3); in accord with the expectation that negative or positive hydrostatic pressures would augment or diminish the fluorescein efflux from the sucrose-gap chamber. In these experiments a diffusion profile was first measured in the absence of a hydrostatic pressure between sucrose and Ringer's solution test node (○). Then a positive or negative pressure was applied across the partition by raising (●) or lowering (×) the level of fluid in the sucrose pool by 3 mm.

In some experiments color photographs of the muscle segment in the test node were taken before and after perfusion of the sucrose pool with 5-mM fluorescein solution. The intense green fluorescence of Na^+-fluorescein could be easily distinguished from the blue background color due to the 450-nm light necessary to excite the dye. The results once again confirmed the more quantitative measurements illustrated in Figs. 1–3.

DISCUSSION

Optical measurements of steady-state diffusion profiles with fluorescein show that dye concentration diffusing in

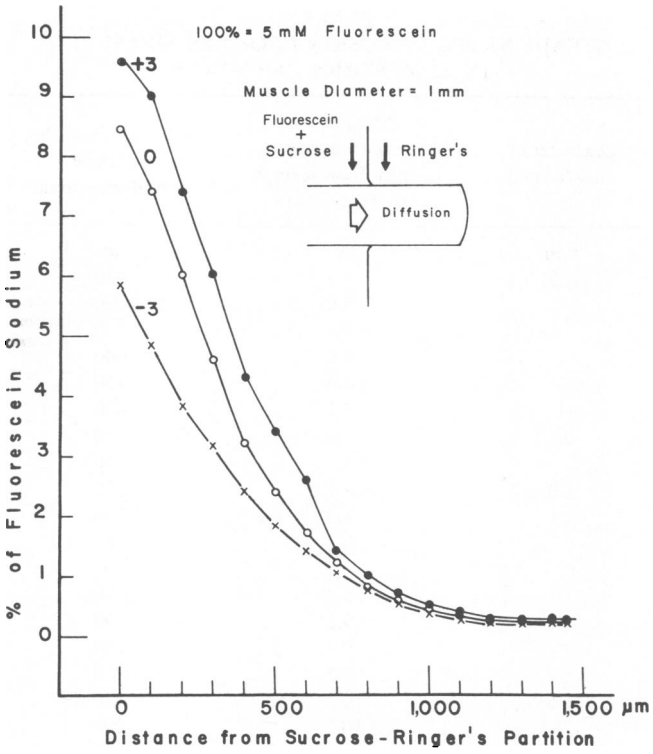

FIGURE 3 Effect of hydrostatic pressure on steady-state diffusion profiles in the extracellular space of a 1-mm diameter muscle bundle in the nonfluorescent physiological solution. Compared with the control state (o), the muscle fluorescein concentration was increased (•) or decreased (×) by raising or lowering the level of the solution in the sucrose-gap pool by 3 mm relative to the level of Ringer's solution in the physiological compartment.

the cross section of test node (1 mm in diameter and 25-μm wide) near the interface was 8 to 10% of its concentration in the gap. In smaller test nodes (0.3 to 0.5 mm in diameter and length) generally used in voltage-clamp experiments (Morad and Orkand, 1971), the maximum dye concentration at the interface was considerably lower, 4 to 6%, and decreased to its half-maximum value ~400 μm from the partition. Fluorescein diffused passively through the extracellular space of the frog ventricular muscle, such that its steady-state profiles were analogous to profiles of sucrose or sodium. The diffusion profiles could be manipulated by changes in the dimensions of the test nodes and small variations in hydrostatic pressure. Thus, for small test nodes, the intermixing of sucrose and Ringer's solutions across the rubber partition contaminates the extracellular spaces of the muscle by ~5% and does not produce steep concentration gradients of solute on either side of the partition.

In contrast with the present findings, experimental (Kleber, 1973) and theoretical analysis (Atwell and Cohen, 1977) predict that the solute concentrations diffusing through the muscle at the interface would be 50% of its concentration in the bathing medium on the opposite side of the partition. Steep gradients of solutes would thus be established within the extracellular space of the muscle under steady-state conditions. Note that in these studies the role of a mechanical partition and its possible influence on diffusion of solutes across the interface were not considered. In practice, ideal rubber partitions do not exist; thin latex membranes used in this study compress the muscle strip to achieve both mechanical and electrical isolation of the physiological and sucrose nodes. Latex diaphragms that are too tight or too loose around the muscle strip sooner or later reduce the magnitude of trans-gap action potentials. Stable trans-gap action potentials of 80 to 100-mV amplitude were used to gauge the proper tightness of the latex diaphragm.

In a more recent theoretical analysis (Lammel, 1981) the squeezing factor of rubber diaphragms on the extracellular space of muscle was taken into account. Such a partition (0.1-mm thick) greatly reduced the predicted solute concentration diffusing just across the partition from 50 to 16%, and reduced the steepness of concentration gradients by increasing the space constant (λ) from 150 to 290 μm, (Lammel, 1981; Fig. 7). Our findings of ~10% contamination and λ = 290 μm (for 1-mm diameter muscle) are in excellent agreement with these theoretical predictions, considering the complex geometry of the extracellular spaces, the variations among cardiac preparations, and the assumptions inherent to mathematical modeling.

In principle, the passive diffusion of all membrane impermeant molecules must produce identical diffusion profiles under steady-state conditions (Atwell and Cohen, 1977). The following findings are consistent with the assertion that fluorescein diffuses freely in the extracellular space of frog ventricular muscle. (*a*) The fluorescence profiles measured 5 min after the addition of dye in the sucrose gap were found to be stable for hours; (*b*) the fluorescence profiles in the test nodes were reversible and could be abolished within 5–10 min by washing the sucrose node with dye-free solution and reinstated by the addition of dye in the gap; (*c*) doubling the dye concentration in the gap doubled the fluorescence profile without altering its shape; (*d*) the diffusion profiles depended on the diameter of the muscle strips and were manipulated by applying small hydrostatic pressures across the partition. These findings suggest that Na^+-fluorescein diffuses rapidly through the extracellular space of the tissue, with little or no binding to, or permeating across the sarcolemma; and that its fluorescence intensity is linear with dye concentration. Thus, the steady-state diffusion profiles of sucrose, sodium and fluorescein were considered identical.

Our studies show that Na^+-fluorescein provide a simple and sensitive optical method to determine the steady-state diffusion profiles of solutes in the extracellular spaces of multicellular preparation. In contrast with other methods to measure diffusion profiles such as radioisotope and ion-selective microelectrodes, this approach can be easily applied to any sucrose-gap chamber where the test node

can be photographed or be observed with the naked eye. The results suggest that the extent of interdiffusion of solutes can be rapidly estimated and manipulated during or prior to a voltage-clamp experiment regardless of the type of preparation or the physical configuration of the sucrose-gap chamber.

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